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ABSTRACT

Under this USDOE-NETL contract, the bacterium *Pseudomonas fluorescens* strain CL0145A (*Pf*-CL0145A) is being developed as a biocontrol agent for zebra mussels *(Dreissena polymorpha* and *Dreissena bugensis*) that infest water pipes in power plants. Progress was made in the following areas during this reporting period:

- A 12-hr treatment at 100 ppm was carried out at the Rochester Gas and Electric (RG&E) Russell Power Station to evaluate the ability to kill zebra mussels in service water. Treatment with *Pf*-CL0145A resulted in an overall mean kill of 69% in *D. bugensis* and 84% in *D. polymorpha*. These mortalities are the highest yet achieved in service water experiments with *Pf*-CL0145A at this RG&E facility. Although not achieving the desired >95% mussel kill, these results were comparable to the moderate levels of mortality that would also have been achieved by chlorination at the same treatment water temperature (17ºC). The overall solution to achieving higher mussel kill, however, is through a genetic approach wherein each bacterial cell is enhanced to produce more toxin. Efforts are now underway to sequence the genome of *Pf*-CL0145A – the first step in this engineering process.
- Advancements in the development of the fermentation medium and culturing protocol provided sufficient quantities of toxic *Pf*-CL0145A cells for use in field trials.
- The development of an effective dead-cell formulation of *Pf*-CL0145A is necessary to minimize possible non-target effects. The increased biomass and volume of *Pf*-CL0145A cells produced in our multiple 100-L fermentation runs revealed that the protocols that were previously developed for smaller amounts of *Pf*-CL0145A cell suspension were less effective on larger quantities. Herein we report that a new method has been developed to produce standardized frozen blocks of suspended *Pf*-CL0145A cells that allowed for effective and consistent penetration of ionizing radiation. This results in high levels of *Pf*-CL0145A cell kill without substantial loss in toxicity to mussels. The described protocol for irradiation of the cell suspension is scalable to larger fermentation volumes that will produce larger quantities of cells.
- Before pipes can be treated, frozen blocks of cell suspension are thawed to produce a homogeneous liquid cell suspension that is held for up to 48 hr before use. Herein we report experiments that demonstrated that the thawed cell suspension can be stored at 0ºC for at least 48 hr without loss in toxicity against mussels. These results helped to further characterize cell toxicity persistence at the selected temperature (0ºC) and provided assurance that there was a degree of flexibility as to when the bacteria could be thawed prior to pipe treatment.

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EXECUTIVE SUMMARY

Use of the bacterium *Pseudomonas fluorescens* strain CL0145A (*Pf*-CL0145A) represents a potential alternative to the current use of polluting biocides for control of zebra mussel infestations in power plant water pipes. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent so that it will be feasible to use it against the two zebra mussel species infesting North American power plants, *Dreissena bugensis* and *D. polymorpha*. During the last six months, research efforts focused on the following three activities:

1. DEMONSTRATION OF EFFICACY IN CONTROL OF ZEBRA MUSSELS IN POWER PLANT SERVICE WATER.

A 12-hr *Pf*-CL0145A experimental treatment at 100 ppm at 17ºC was carried out at the Rochester Gas and Electric (RG&E) Russell Power Station to evaluate the ability to kill zebra mussels in service water. Treatment with *Pf*-CL0145A resulted in an overall mean kill of 69% to *D. bugensis* and 84% to *D. polymorpha*. These mortalities are the highest yet achieved in service water experiments with *Pf*-CL0145A at this RG&E facility. Although not achieving the desired >95% kill, these levels of mortality were comparable to what could have been achieved by chlorination at the same 17ºC treatment water temperature. A solution to achieving higher mussel kill, however, is through a genetic approach wherein each bacterial cell is enhanced to produce more toxin. Efforts are now underway to sequence the genome *Pf*-CL0145A – the first step in this engineering process.

2. DEVELOPMENT AND SCALE-UP OF A NEW FERMENTATION MEDIUM AND PROCESS FOR INCREASED BIOMASS PRODUCTION WITH EFFICACY AGAINST BOTH ZEBRA MUSSEL SPECIES.

Laboratory tests conducted throughout last year indicated that *D. bugensis* was less susceptible than *D. polymorpha* to kill by *Pf*-CL0145A. In our last technical report (R41909R03) we documented the successful development of a culture medium that produced *Pf*-CL0145A cells of increased toxicity to *D. bugensis*. This medium, however, contained only the minimum required nutrients for culture growth and resulted in low levels of biomass production (i.e., cell yield). Using this minimal medium, the production of sufficient quantities of *Pf*-CL0145A cells required for power plant trials was unrealistic unless higher biomass production could be achieved. This would have to be accomplished first at the flask-level (ca. 25 mL) and then in larger culture volumes (e.g., up to 100 L). Herein we report the successful development of a new fermentation medium that produces cells of both high toxicity and yield – a major advance for the project.

3. DEVELOPMENT OF AN EFFECTIVE FREEZING, IRRADIATING, AND THAWING PROTOCOL FOR *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A CELLS.

- Irradiation: Treating with dead bacterial cells minimizes the risk of adverse impact on non-target organisms, and we have previously indicated that ionizing radiation is a useful method to kill *Pf*-CL0145A. These prior studies typically required processing relatively small volumes of bacterial biomass. Herein we report that methods have been developed to process large volumes of *Pf*-CL0145A cell suspensions (e.g., from 100-L fermentations) in which almost all of the bacterial cells are inactivated by ionizing radiation without losing substantial efficacy against zebra mussels.
- Storage: Ultra-low temperature (–80ºC) freezing of *Pf*-CL0145A cell suspensions is currently the most effective method for long-term storage of the bacteria. There are situations when it is necessary to thaw the bacteria for up to 2 days prior to their use in treating pipes. We have been concerned that the *Pf*-CL0145A cells may lose toxicity during this thawed storage period, rendering the cells less effective. Laboratory experiments conducted during this reporting period, however, have demonstrated that live or killed cell suspensions can be thawed and stored at 0ºC for at least 2 days (48 hr) without reducing their efficacy against zebra mussels. These results have been reassuring as they permit flexibility in setting up experiments.

INTRODUCTION

Coal-fired power plants within North America need an effective, economical, and non-polluting technique for managing infestations of zebra mussels within their facilities. Due to a lack of options, many facilities have relied on the use of broad-spectrum, chemical biocides for control of these freshwater mussels. However, biocide treatments, such as continuous chlorination for three weeks, are widely regarded as environmentally unacceptable because they can result in the formation of potentially carcinogenic substances. Use of the bacterium *Pseudomonas fluorescens* strain CL0145A (*Pf*-CL0145A) represents a potential alternative to the use of polluting biocide treatments and is the leading candidate in the world for the biological control of these macrofouling mussels. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent so that it will be feasible to use against the two zebra mussel species infesting North American power plants, *Dreissena bugensis* and *D. polymorpha*. During the last six months, progress was made in the following three research areas:

1. DEMONSTRATION OF EFFICACY IN CONTROL OF ZEBRA MUSSELS IN POWER PLANT SERVICE WATER.

Traditional laboratory testing is centered on experiments in aerated containers with recirculating water because they are easily managed, can evaluate many variables at once, and are much less time consuming to conduct. Mussels in power plants, however, are under flow-through conditions in pipes, so it is important to demonstrate that *Pf*-CL0145A cells are capable of achieving high mussel kill under more realistic, i.e., flow-through, pipe conditions. Toward this end, an experiment was conducted in the service water system at RG&E's Russell Station power plant.

2. DEVELOPMENT AND SCALE-UP OF A NEW FERMENTATION MEDIUM AND PROCESS FOR INCREASED BIOMASS PRODUCTION WITH EFFICACY AGAINST BOTH ZEBRA MUSSEL SPECIES.

We have previously reported that *Pf*-CL0145A cells harvested from cultures using a production fermentation medium, FM1, which was previously developed against *D. polymorpha*, were found to be less efficacious against *D. bugensis* in laboratory trials. However, as indicated in our last technical report (R41909R03), cells harvested from a semi-defined minimal medium that was under development, ASM1, achieved high mortality against both species. Since the biomass production in ASM1 cultures was 7.5 times lower than that achieved in FM1, large-scale treatments were unrealistic using ASM1. Therefore, experiments were designed to increase the biomass in ASM1 without reducing cell toxicity. Herein we report the successful development of a new medium, ASM2, in which biomass yield was increased 4.3-fold over ASM1 in shake-flasks. In addition, ASM2 was further developed into a new production fermentation medium, FM2, in which CL0145A cultures were successfully scaled-up from shake-flasks to 0.5-L and 100-L fermentors without loss in efficacy to mussels and with increased biomass production.

3. DEVELOPMENT OF AN EFFECTIVE FREEZING, IRRADIATING AND THAWING PROTOCOL FOR *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A CELLS.

Pf-CL0145A cells must be killed prior to their use in treating power plants to minimize possible non-target effects. In technical report R41909R01, we documented the development of an ionizing radiation protocol capable of killing relatively small quantities of *Pf*-CL0145A cells. Since that time, however, the increased volume of *Pf*-CL0145A cells produced in multiple 100-L fermentation runs has required that this protocol be revised. In killing *Pf*-CL0145A cells, the quantity of radiation that the cells receive is critically important as excessive levels can destroy their toxicity to zebra mussels. Herein we report the development of a new protocol for freezing and irradiating larger volumes of *Pf*-CL0145A cells. This new protocol continues to use frozen blocks of *Pf*-CL0145A cells, but with uniform, defined thickness to ensure adequate penetration of the desired radiation dosage without substantial loss in their toxicity.

Before a power plant treatment begins, the irradiated frozen blocks of *Pf*-CL0145A cells must be thawed so that the cells can be injected into the pipes as a liquid suspension. To allow flexibility in setting up an experiment, the length of time from thawing the cell suspension to actual pipe treatment can be up to 48 hr. In this reporting period, experiments were conducted that demonstrated that the thawed irradiated cell suspension can be held at 0ºC for at least 48 hr without loss in toxicity.

EXPERIMENTAL

The following is an overview of the materials and methods used to conduct the tests reported herein:

1. DEMONSTRATION OF EFFICACY IN CONTROL OF ZEBRA MUSSELS IN POWER PLANT SERVICE WATER.

The following is a general outline of the methodology employed in the trial conducted within the RG&E Russell Power Station to assess the ability of *Pf*-CL0145A to kill zebra mussels in service water piping:

- Bacterial production: *Pf*-CL0145A was cultured at the University of Iowa's Center for Biocatalysis and Bioprocessing within a 100-L fermentation unit following our standardized protocol. The harvested bacterial mass was subsequently frozen in blocks at -80ºC and irradiated to kill the cells. Frozen blocks were thawed within 48 hr prior the test, with the aqueous cell suspension held at ~1ºC until use.
- Preparation of mussels: Prior to testing within the plant, mussels were held in acrylic pipes under flow-through conditions within a research trailer on the power plant grounds (Fig. 1). The trailer receives water diverted from the plant's intake pipe and mussels were held at ambient Lake Ontario water temperatures. Although zebra mussels at this power station are almost exclusively *D. bugensis*, it was of interest to also evaluate the effectiveness of *Pf*-CL0145A against *D. polymorpha* in this test. *D. polymorpha* were thus field collected from Irondequoit Bay (Rochester, NY) and Seneca Lake (Geneva, NY) (Fig. 2). To obtain *D. bugensis* for use in the test, individuals of that species were selected from those that naturally had populated the flow-through water system within the trailer.

Figure 1. The Rochester Gas & Electric Company has provided use of a research trailer (foreground) with continuously flowing intake water on the grounds of their Russell Power Station. The trailer contains areas for small-scale pipe tests as well as holding areas for maintaining mussels both before and after conducting experiments within the plant.

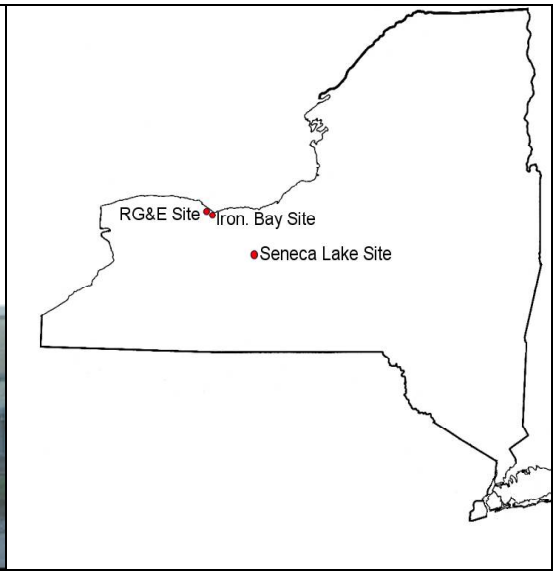

Figure 2. The locations of mussel collections for the in-plant efficacy testing of *Pf*-CL0145A.

- Bacterial treatment: Acrylic pipes containing mussels were placed along their longest side inside a ~120 liter plastic biotank held under flow-through conditions at 5.4 L/min directly from the service water system inside the power plant 24 hr before the experimental treatment. The plastic

biotanks were very similar to biotanks the power plant currently uses for chlorine efficacy monitoring (Fig. 4). A metering pump, similar to the those already used by the plant personnel for chlorination, was used to inject a 1°C aqueous suspension of *Pf*-CL0145A directly into the stream of flowing service water at the beginning of a ca. 30-m run of service water piping (Fig. 3). The bacterial injection resulted in a 100 ppm treatment concentration for 12 hr which had to pass through the 30 m of service water piping before reaching the exposed mussels. The control mussels were contained in an identical biotank receiving service water flow from a valve just upstream of the bacterial injection point. After treatment, mussels were removed from the power plant and placed back in the flow-through conditions inside the research trailer.

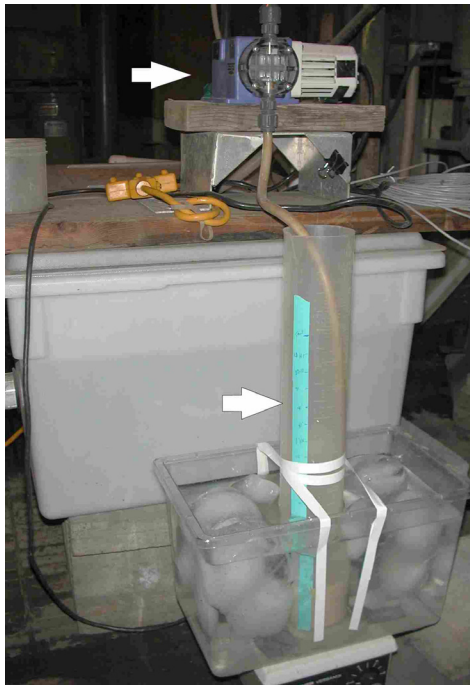

Figure 3. *Pf*-CL0145A application configuration. A metering pump (indicated by the uppermost arrow) injects bacteria into the service water piping. The graduated cylinder (lower arrow) contains the bacterial suspension that is constantly being stirred and chilled in the ice water bath (~1°C).

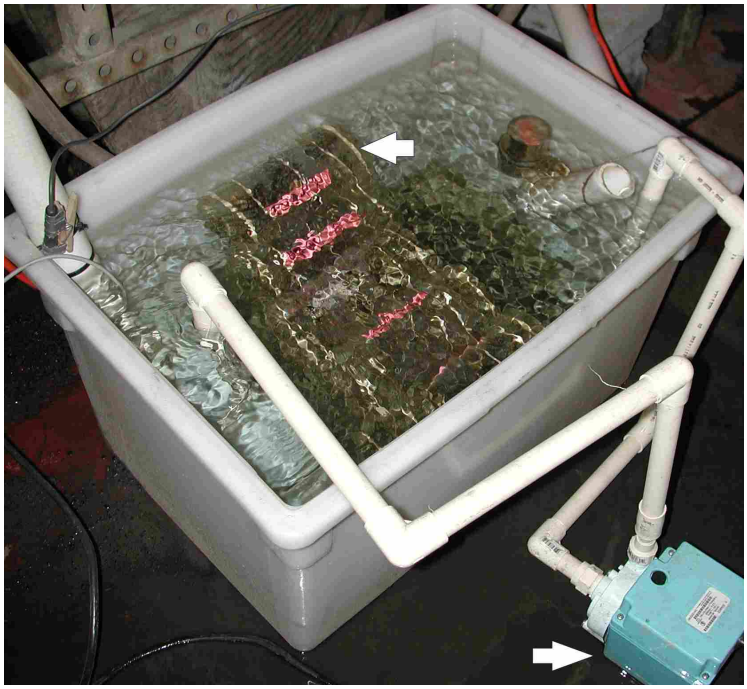

Figure 4. Typical biotank configuration. The mussels are contained in acrylic pipes with netting over each end (uppermost arrow). To ensure adequate movement of water through the pipes, a small blue pump is recirculating water inside the biotank (lower arrow).

- Water quality monitoring: During the 12-hr treatment, the optical density of the treated service water was monitored to confirm that the bacterial treatment concentrations were near the target 100 ppm. The mean water temperature during the 12-hr treatment was 17°C, the mean pH was 7.5, and mean oxygen concentration was 9.2 ppm. Mussels were held after treatment at ambient Lake Ontario temperatures (Fig. 11).

2. DEVELOPMENT AND SCALE-UP OF A NEW FERMENTATION MEDIUM AND PROCESS FOR INCREASED BIOMASS PRODUCTION WITH EFFICACY AGAINST BOTH ZEBRA MUSSEL SPECIES.

The following is a general outline of the methodology employed in these culturing tests:

- Shaken seed cultures: 250-mL Erlenmeyer flasks containing 25 mL of buffered tryptic soy broth (bTSB) were inoculated with 0.4 mL of stock culture and shaken at 200 rpm at 26±1ºC for 24 hr.

- Shaken flask cultures: Replicate flasks containing 35 mL of the experimental culture medium were inoculated at a 1% (v:v) concentration with the 24-hr seed culture. Flasks were shaken at 200 rpm at 26±1ºC for 24 hr.
- 0.5-L experimental fermentations: Experimental fermentations were conducted using six 0.5-L (working volume) fermentors (Appropriate Technical Resources, Inc.) (Fig. 5). Medium components were modified in duplicate fermentors to aid in the development of the new fermentation medium (FM2) and protocol. Individual 0.5-L fermentors were inoculated at a 1% (v:v) concentration with the 24-hr seed culture. Sub-samples from the cultures were removed over the culture period from 0 to 24 hr to determine the optimal harvest time.
- 100-L production fermentations using FM2: Production fermentations were contracted with the Center for Biocatalysis and Bioprocessing at The University of Iowa using a100-L fermentor (Biostat D100) (Fig. 6).

Figure 5. 0.5-L fermentors used for fermentation medium and process development.

Figure 6. 100-L fermentor used for production runs using new fermentation medium (FM2).

- Production of cell fraction (CF): The final whole culture (FWC) from each culture was centrifuged and the pellets were combined to produce a common pellet. Cell pellets were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \cdot 6H_2O$ in deionized water).
- Preparation of CF: Mean dry bacterial cell mass/mL for each CF was calculated from 2-1.0 mL desiccated subsamples using a Denver Instruments balance. The amount of inoculum needed to treat at the targeted concentration was based on the mean dry bacterial cell mass/mL.
- Preparation of mussels: *Dreissena* sp. were collected from various locations, brought to the testing site, sieved, and stored until testing began. Collection sites included: the Mohawk River (Crescent, NY), Irondequoit Bay (Rochester, NY), and the research trailer receiving Lake Ontario water at Rochester Gas & Electric (Rochester, NY).
- Standard laboratory bioassays of mussels with CF: The day before treatment, mussels were picked, placed into testing containers (either micro-chambers or testing jars) containing 5 or 100 mL of aerated hard water (Peltier and Weber, 1985), respectively, and allowed to attach overnight. The morning of treatment, unattached mussels were replaced with attached mussels from an extra dish. At least one hour before treatment, the testing containers were filled with fresh aerated hard water (10 mL for micro-chambers, 500 mL for testing jars), set up with aeration, and labeled (Figs. 7 and 8). Mussels were exposed for the treatment period (24 hr), then the fluid was poured off and mussels were collected in clean plastic dishes with fresh, oxygenated hard water to be examined for mortality. Mussels were held in the dishes for an additional 6 (micro-chambers) or 9 (testing jars) days changing the water and scoring mortality each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

Figure 7. Three replicate micro-chambers containing zebra mussels with aeration.

Figure 8. Treatment of mussels in standard testing jar bioassay.

3. DEVELOPMENT OF AN EFFECTIVE FREEZING, IRRADIATING AND THAWING PROTOCOL FOR *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A CELLS.

The following is a general outline of the methodology employed in these culturing tests:

• Production of cell fraction (CF): The final whole culture (FWC) from each 100-L production fermentation was centrifuged at The Center for Biocatalysis and Bioprocessing and the pellets were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \cdot 6H_2O$ in deionized water). The CF was dispensed into zip-lock freezer bags and laid flat in an ultrafreezer (-80ºC). Frozen blocks were packed in coolers with dry ice and shipped to the Cambridge Laboratory.
• Packaging of CF and freezing: Upon receipt at the Cambridge Laboratory, the frozen blocks were thawed and the products from multiple 100-L fermentations were combined in a total volume of 10.125 L as a single homogeneous suspension. To produce blocks of uniform thickness, ca. 375 mL of the cell suspension was poured into each small zip-lock bag (Fisher #AJS31798C, 20x30 cm) and laid on a sheet of acrylic in a large styrofoam cooler with dry ice. Two such bags were laid side-by-side on one sheet of acrylic. Another layer of bags were laid on top of the first layer, then another sheet of acrylic was placed on top, followed by another layer of bags, etc. (Fig. 9). The average thickness of each frozen block was 0.925 cm (Fig. 10).
• Killing CL0145A cells with irradiation: Frozen blocks of cells were transported to the E-Beam facility in Cranbury, NJ on dry ice. The <1-cm-thick frozen blocks were arranged in a single layer and passed by the irradiating beam to be exposed to actual irradiation dosages of 0.3, 0.95 and 1.2 Mrad. The actual exposure was determined from radiation sensitive film exposed during each E-Beam treatment.
• Standard plate counts (SPCs): SPCs were performed to determine the number of live cells remaining after irradiation. A non-irradiated, live sample was thawed just before treatment; and both samples were plated and bioassayed to determine the relative level of cell kill and toxicity following irradiation treatment.

• Evaluation of toxicity of CF: Toxicity was determined using similar methods as described in the section above. Relative toxicity of irradiated CF was assessed by calculating the ratio between mean angular transformed mortalities of irradiated vs. non-irradiated CF.
• Storage of thawed irradiated *Pf*-CL0145A cell suspension: The thawed *Pf*-CL0145A cell suspension was stored in an ice bath in a cooler to maintain temperature at 0ºC throughout the storage period.

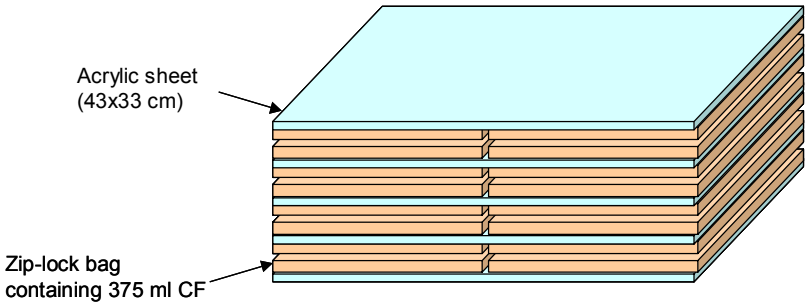

Figure 9. Schematic drawing of stacked zip-lock bags containing suspended *Pf*-CL0145A cells with acrylic sheets used to produce frozen blocks with uniform average thickness of 0.925 cm.

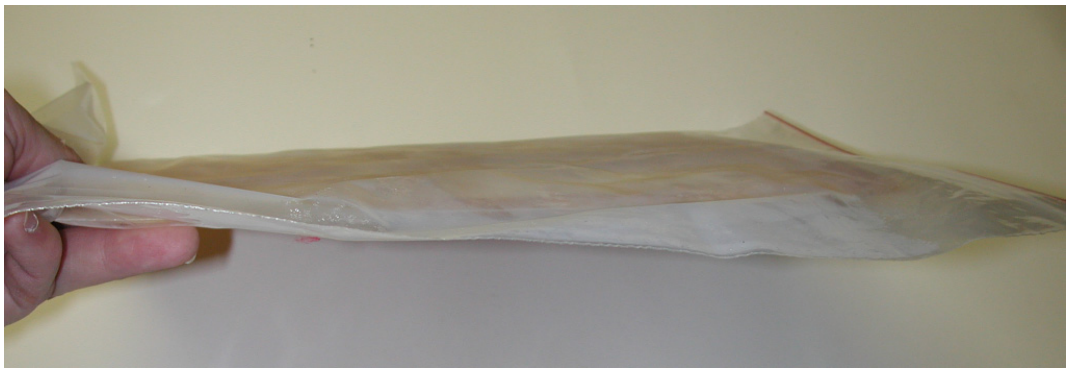

Figure 10. Zip-lock bag containing frozen suspended *Pf*-CL0145A cells prepared for irradiation (0.925 cm thick).

RESULTS

This section gives an overview of the progress made during the six-month reporting period in both planning and conducting experiments:

1. DEMONSTRATION OF EFFICACY IN CONTROL OF ZEBRA MUSSELS IN POWER PLANT SERVICE WATER.

The bacterial suspension of *Pf*-CL0145A was successfully delivered into the pressurized service water system within the RG&E Russell Power Station using a metered pump similar to that already operated by RG&E personnel for chlorination. The 12-hr treatment at 100 ppm resulted in mean mortalities of 69% of *D. bugensis* and 84% of *D. polymorpha*. A summary of mortality data is provided in Table 1 and Figure 11.

Table 1: Summary of mussel mortalities scored over 289 days.* Subscripted letters indicate statistical differences between treatment groups ($p<0.05$).

Mussels	% Mortality	Mean % mortality (± SD)	Mean angular transformed mortality (±SD)
Seneca Lake *D. polymorpha*	82, 89, 88	86.1 ± 3.5	$1.177 \pm 0.060_a$
Irondequoit Bay *D. polymorpha*	79, 87, 82	82.7 ± 4.0	$1.146 \pm 0.047_a$
RG&E *D. bugensis*	66, 69, 73	69.2 ± 3.5	$0.933 \pm 0.032_b$

*Mean ±SD control (untreated) mussel mortalities were: Seneca Lake *D.p.* = 12.4 ± 2.1%; Irondequoit Bay *D.p.*= 6.6 ± 3.5%; RG&E *D.b.* = 4.1 ± 1.8%.

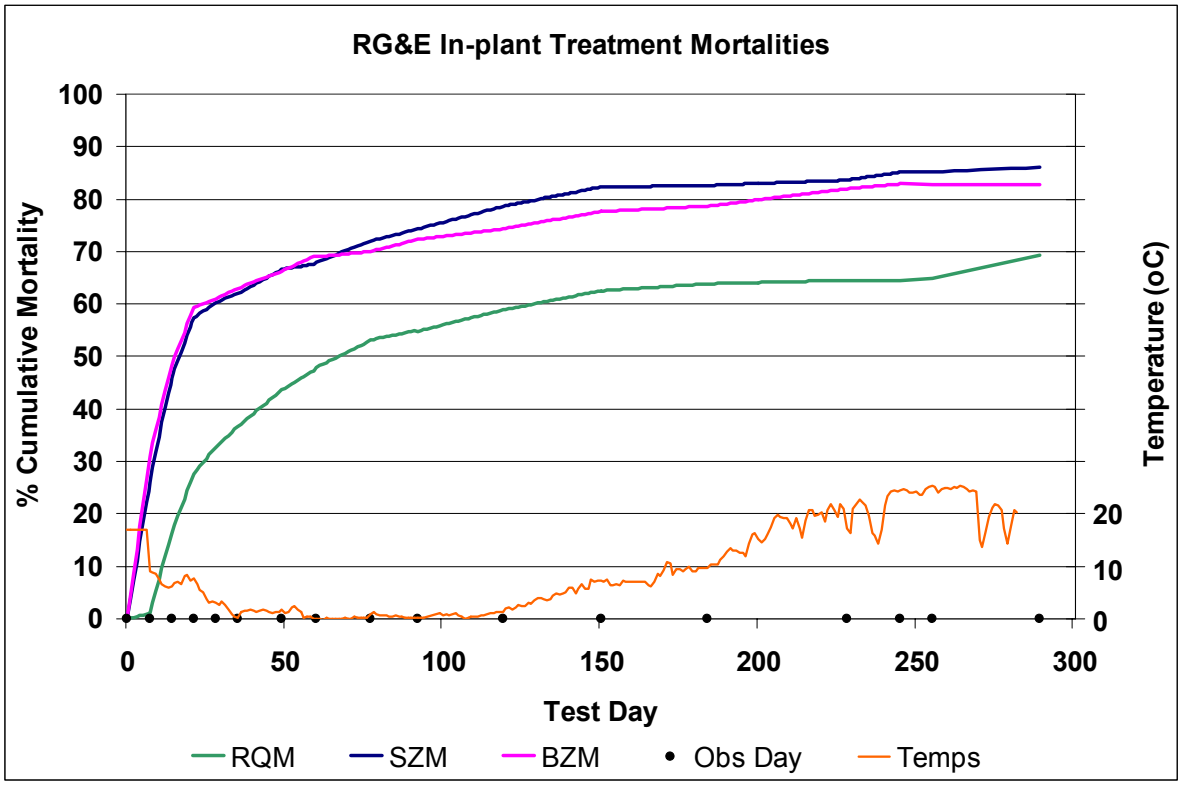

Figure 11. Graphical summary of pipe mortality data. RQM = RG&E *D. bugensis*, SZM = Seneca Lake *D. polymorpha*, BZM = Irondequoit Bay *D. polymorpha*, Obs Day = days that mortality observations were performed, Temps = mussel holding temperature.

2. DEVELOPMENT AND SCALE-UP OF A NEW FERMENTATION MEDIUM AND PROCESS FOR INCREASED BIOMASS PRODUCTION WITH EFFICACY AGAINST BOTH ZEBRA MUSSEL SPECIES.

We previously reported our success at killing *D. bugensis* when treating with *Pf*-CL0145A cells harvested from our experimental culture medium, ASM1 (Figs. 12 and 13). A limitation in the use of ASM1 as a culture medium for the production of high quantities of *Pf*-CL0145A cells was that while *Pf*-CL0145A cells harvested from this medium achieved high toxicity against both species of zebra mussel, the yield of cell biomass produced in ASM1 cultures was much lower than our previously developed fermentation medium (FM1). ASM1 was developed as a minimal medium for experimental purposes in our fermentation improvement testing, and our focus was to first determine the nutritional components required to increase cell toxicity rather than high levels of cell biomass. Large-scale flow-through treatments would be unrealistic given the low level of cell biomass produced in this experimental minimal

medium. Therefore, a series of experiments were conducted to increase the biomass yield in this minimal medium to produce a new fermentation medium (FM2) in which large quantities of toxic *Pf*-CL0145A cells could be produced for scheduled power plant experiments. Biomass production was first increased in shake-flask cultures through the modification of the peptone components in ASM1 to produce a new experimental minimal medium with higher biomass yield, ASM2. This led to the development of a new fermentation medium for production runs, FM2. Biomass production in FM2 was increased approximately two-fold over ASM2 in the shake-flask cultures to ca. 4 g/L in 0.5-L fermentors (Fig. 14). *Pf*-CL0145A cultures in 0.5-L fermentors using FM2 achieved similar toxicity to zebra mussels as cells from shake-flask cultures (Fig. 15). Therefore, a fermentation protocol was developed in the 0.5-L Sixfors fermentors to scale-up production in 100-L fermentors. Mean biomass production was 4.5 g/L from three recent 100-L runs and similar toxicity was achieved compared to *Pf*-CL0145A cells harvested from 0.5-L fermentors and shake-flasks (Fig. 15).

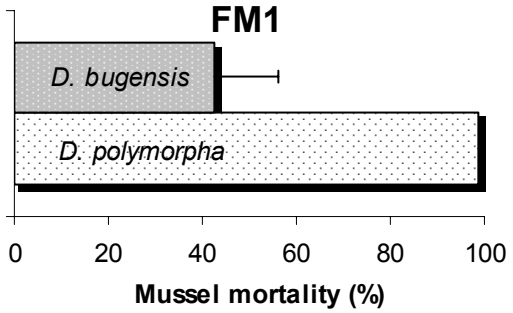

Figure 12. Both mussel species were exposed to CF harvested from cultures in FM1 at 100 ppm (dry weight *Pf*-CL0145A cells/unit volume) for 24-hr. Final mean % mortality was assessed after 10 days.

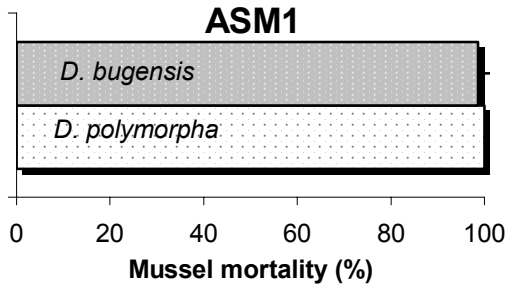

Figure 13. Both mussel species were exposed to CF from cultures in ASM1 at 100 ppm (dry weight *Pf*-CL0145A cells/unit volume) for 24-hr. Final mean % mortality was assessed after 10 days.

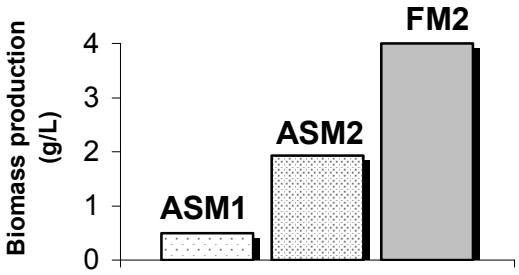

Figure 14. Biomass production was increased over 7-fold from ASM1 to FM2, producing sufficient biomass for use in larger-scale fermentations.

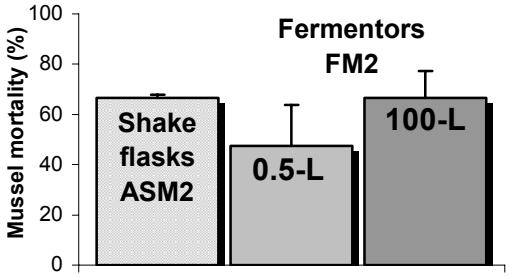

Figure 15. Bioassay mortality results after treating *D. polymorpha* at 25 ppm for 24 hr and assessing final mean % mortality after 10 days.

3. DEVELOPMENT OF AN EFFECTIVE FREEZING, IRRADIATING AND THAWING PROTOCOL FOR *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A CELLS.

The protocol for freezing the *Pf*-CL0145A cell suspension in thin blocks (0.925 cm thick) appeared to allow for more effective penetration of the irradiating beam at E-Beam than exposure of the thicker (2.54 cm) blocks (Table 2). Irradiation exposures of 0.3 and 1 Mrad reduced active *Pf*-CL0145A cells by approximately 3-log and 9-log more when the thin blocks were exposed compared to the thicker frozen blocks, respectively. The linear regression analysis derived from the standard plate count (SPC) and mortality data described in Figure 16 will aid in selecting the level of irradiation exposure and predict the corresponding expected loss in cell toxicity to zebra mussels as well as the reduction in active cells. For example, to achieve ca. 7-log reduction in active cells (99.99999% reduction), the 0.925 cm thick frozen

block of *Pf*-CL0145A cells should be exposed to ca. 0.6 Mrad of irradiation with a corresponding predicted loss in efficacy of 10-15%. This protocol will be valuable for preparing *Pf*-CL0145A cells in liquid formulation to obtain consistent levels of active cell reduction in future trials.

Table 2. Effectiveness of E-Beam irradiation on frozen blocks of *Pf*-CL0145A cell suspension produced by two different protocols, resulting in "thick" blocks (ca. 2.54 cm) or "thin" blocks (0.925 cm).

E-Beam dosage	Thick block	Thin block
	% Cell reduction	
0.3 Mrad	87.0%	99.9975%
1 Mrad*	97.0%	99.999999980%
	Active cells remaining in CF (cfu per mg dry weight)	
0.3 Mrad	4.4×10^8	2.9×10^5
1 Mrad*	1.0×10^8	0.14

* The thin blocks were exposed to 0.95 Mrad.

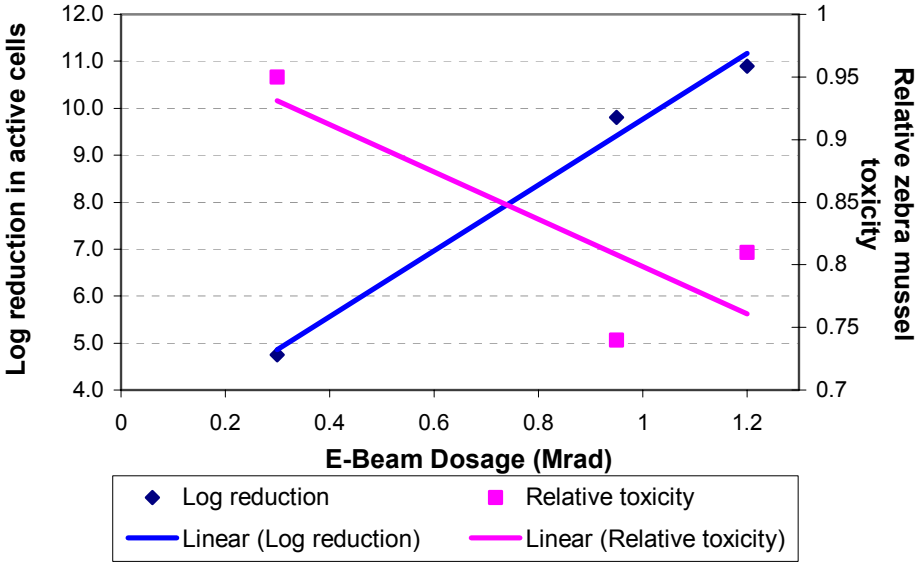

Figure 16. Summary of irradiation effects on cell kill and toxicity to zebra mussels from irradiated suspended *Pf*-CL0145A CF from multiple 100-L fermentation batches.

Another aspect of product development is storage. Before treatment application, the irradiated frozen blocks of cell suspension are thawed to produce a homogeneous liquid cell suspension. The length of time from thawing the cell suspension to application should be flexible within at least 2 days. The relative toxicity data presented in Figure 17 suggest that thawed irradiated *Pf*-CL0145A cells can be stored at 0ºC for at least 48 hr without losing toxicity. These results provide a certain level of flexibility in the handling the bacteria prior to pipe treatment.

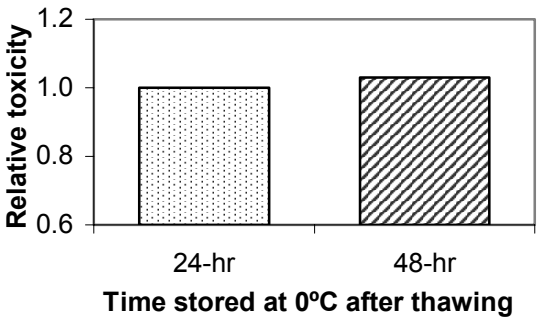

Figure 17. Maintenance of efficacy during storage of irradiated *Pf*-CL0145A cells: Relative toxicity of irradiated *Pf*-CL0145A cells after thawing and storing for 24 or 48 hr at 0ºC compared to toxicity of cells thawed and applied without storage.

DISCUSSION

1. DEMONSTRATION OF EFFICACY IN CONTROL OF ZEBRA MUSSELS IN POWER PLANT SERVICE WATER.

The data presented in this report represent the highest levels of mortality achieved to date under actual power plant service water flow-through conditions at RG&E. *Dreissena polymorpha* and *D. bugensis* mortalities were 83% and 69%, respectively. While the levels of mussel kill were not as high as targeted (>95% kill), this may be due to the cold water conditions in which the mussels were held post-treatment (typically <10C), but this likely does not fully account for the lower than desired kill among both mussel species. However, these levels of mortality were comparable to what might have been expected after chlorination at the 17ºC treatment water temperature. Higher mussel kill could be achieved using a genetic approach wherein each bacterial cell is enhanced to produce more toxin. Efforts are now underway to sequence the genome *Pf*-CL0145A – the first step in this genetic enhancement process.

2. DEVELOPMENT AND SCALE-UP OF A NEW FERMENTATION MEDIUM AND PROCESS FOR INCREASED BIOMASS PRODUCTION WITH EFFICACY AGAINST BOTH ZEBRA MUSSEL SPECIES.

During this reporting period, the culture medium was modified to result in production-level quantities of toxic *Pf*-CL0145A cell biomass to be used in field trials and the successful scale-up of the fermentation protocol to 100-L. One of our next culturing tasks will be to produce an economic commercial fermentation medium by omitting or replacing components of the current medium with less expensive components while maintaining high levels of biomass production and efficacy of the *Pf*-CL0145A cell-based product.

3. DEVELOPMENT OF AN EFFECTIVE FREEZING, IRRADIATING AND THAWING PROTOCOL FOR *PSEUDOMONAS FLUORESCENS* STRAIN CL0145A CELLS.

The development of an effective dead-cell formulation of *Pf*-CL0145A is necessary to minimize possible non-target effects. The increased biomass and volume of *Pf*-CL0145A cells produced in multiple 100-L fermentation runs, or larger fermentation runs planned for the future, revealed that the protocols that were previously developed for smaller amounts of *Pf*-CL0145A cell suspension were less effective on larger quantities. During this reporting period, a new method was developed to produce standardized frozen blocks of suspended *Pf*-CL0145A cells that allowed for effective and consistent penetration of ionizing radiation, thus resulting in high levels of cell kill without substantial loss in cell toxicity against mussels. Multiple irradiation dosages ranging from 0.3 to 1.2 Mrad allowed us to predict the desired dosage of irradiation required for desired levels of *Pf*-CL0145A cell inactivation while minimizing reductions in their toxicity. The described protocol for irradiation of the cell suspension is scalable to larger fermentation volumes that will produce larger volumes of cells.

Another aspect of product development is storage. Before treatment application, the irradiated frozen blocks of cell suspension are thawed to produce a homogeneous liquid cell suspension. The length of time from thawing the cell suspension to application should be flexible within at least 2 days. In this reporting period, experiments were conducted that demonstrated that the thawed irradiated cell suspension can be stored at 0º for at least 48 hr without loss in toxicity against mussels. These two formulation developments for effective *Pf*-CL0145A cell kill and short-term storage are essential steps in the development of this bacterium as a commercial product. The definition of the handling and storage characteristics of the current *Pf*-CL0145A cell-based liquid formulation will aid in the development of a commercially viable product in the future.

CONCLUSIONS

The facility trial conducted during this reporting period demonstrated that moderate levels of mortality (ca. 70-85%) could be achieved against both species of zebra mussels in a power plant's service water system. Use of the bacterium as a practical control agent to clear zebra mussel infestations from power plant pipes, however, requires treatments that can consistently achieve >95% kill. If *Pf*-CL0145A is ever going to be commercialized, such moderate levels of mortality are thus not acceptable. The most effective solution to achieving this high mussel kill is through a genetic approach wherein each bacterial cell is enhanced to produce more toxin. The first step in this process is now being planned with the genomic sequencing of *Pf*-CL0145A scheduled for FY 2006.

During this reporting period progress was made: 1) in the development and scale-up of a fermentation process that produces high quantities of *Pf*-CL0145A cells that are effective against both mussel species; 2) in developing a new protocol for irradiation of cell suspensions that is scalable to larger fermentation volumes; 3) in confirming that, following thawing, aqueous cell suspensions, can be held at 0ºC for at least 48 hr without loss in toxicity. These advances will facilitate future use of the bacterium.

REFERENCES

Peltier, W. H. and Weber, C. I. 1985. Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Third edition. U. S. EPA Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio. 216 pp.

Sokal, R. R. and Rohlf, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. Third edition. W. H. Reeman and Company, New York. 887 pp.

TECHNOLOGY AND INFORMATION TRANSFER

This project was highlighted in the following presentation:

Mayer, D. A. and D. P. Molloy. Increased efficacy of *Pseudomonas fluorescens* biocontrol strain CL0145A through the modification of culture media. Annual Meeting of the Society for Industrial Microbiology. August 21-25, 2005. Chicago, IL. (Submitted poster.)